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ORIGINAL PAPER

Autotrophic, sulfur-oxidizing actinobacteria in acidic environments

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Abstract Some novel actinobacteria from geothermal environments were shown to grow autotrophically with sulfur as an energy source. These bacteria have not been formally named and are referred to here as "*Acidithiomicrobium*" species, as the first of the acidophilic actinobacteria observed to grow on sulfur. They are related to *Acidimicrobium ferrooxidans* with which they share a capacity for ferrous iron oxidation. Ribulose bisphosphate carboxylase/oxygenase (RuBisCO) is active in CO_2 fixation by *Acidimicrobium ferrooxidans*, which appears to have acquired its RuBisCO-encoding genes from the proteobacterium *Acidithiobacillus ferrooxidans* or its ancestor. This lateral transfer of RuBisCO genes between a proteobacterium and an actinobacterium would add to those noted previously among proteobacteria, between proteobacteria and cyanobacteria and between proteobacteria and plastids. "*Acidithiomicrobium*" has RuBisCO-encoding genes which are most closely related to those of *Acidimicrobium ferrooxidans* and *Acidithiobacillus ferrooxidans*, and has additional RuBisCO genes of a different lineage. 16S rRNA gene sequences from "*Acidithiomicrobium*" species dominated clone banks of the genes extracted from mixed cultures of moderate thermophiles growing on copper sulfide and polymetallic sulfide ores in ore leaching columns.

Keywords Acidophiles · Actinobacteria · Autotrophy · Sulfur oxidation

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Introduction

Extreme environments continue to be a source of previously unrecognized bacteria. Those utilizing inorganic compounds as energy sources can directly influence the geochemistry of their environments. A large number of uncultured bacteria of the class *Actinobacteria* have been indicated as potential members of the *Acidimicrobidae* subclass through analysis of their 16S rRNA genes. This subclass (Stackebrandt et al. 1997) was created for a ferrous iron-oxidizing acidophile, *Acidimicrobium ferrooxidans* (Clark and Norris 1996). Alignment-independent analysis of 16S rRNA gene sequences confirmed the placement of *Am. ferrooxidans* in a phylogenetically deep-branching sub-group of the *Actinobacteria* (Rudi et al. 2006). *Am. ferrooxidans* has been noted as phylogenetically the closest relative of *Iamia majanohamensis*, which was isolated from sea cucumber and assigned to the *Acidimicrobidae* (Kurahashi et al. 2009), and of *Ilumatobacter fluminis*, which was isolated from an estuary sediment (Matsumoto et al. 2009). The higher taxa of these species, which are apparently related to *Am. ferrooxidans* but which are neither acidophilic nor chemolithotrophic, should be reviewed when future isolations allow further phylogenetic analysis,

as suggested by Matsumoto et al. (2009). Two species of the *Acidimicrobidae* that are more closely related phylogenetically and physiologically to *Am. ferrooxidans* have been described: *Ferrimicrobium acidiphilum* and *Ferrithrix thermotolerans* (Johnson et al. 2009). These acidophilic, ferrous iron oxidizers have lower mol% G+C contents (50–55 mol%) than *Am. ferrooxidans* (68 mol% G+C). They lack the capacity of *Am. ferrooxidans* for autotrophic growth. Autotrophy through operation of the Calvin cycle has only exceptionally been reported for actinobacteria, e.g. in hydrogen-oxidizing *Nocardia opaca* (now *Rhodococcus opacus*) where ribulose bisphosphate carboxylase/oxygenase (RuBisCO) is encoded on a megaplasmid (Ecker et al. 1986; Kalkus et al. 1990).

Here, new isolates related to *Am. ferrooxidans* are discussed in relation to their mostly uncultured relatives and to autotrophy within these actinobacteria. The oxidation of sulfur by one of the new isolates is also described, a feature with relevance to their biogeochemical activity and to their potential for application in ore biomining.

Methods

Strain isolation and growth

Two enrichment cultures were maintained with 1% (w/v) pyrite as substrate and occasional serial culturing at 47°C. One of these mixed cultures was established in 2006 at 58°C with a sample of elemental-sulfur-rich, acidic soil (50–60°C) from the surface (0–2 cm depth) of a geothermal site at Palaeochori Bay of the island of Milos in the Aegean Sea. The second culture was established at 48°C with samples from several sources, which included natural geothermal environments and mineral sulfide and coal mines (Cleaver et al. 2007). This enrichment culture was used previously in studies of metal extraction from mineral sulfides (Dew et al. 1999; Cleaver et al. 2007). Phytagel plates supplemented with ferrous iron and tetrathionate (Clark and Norris 1996) were incubated at 47°C for isolation of single colonies of "*Acidithiomicrobium*" strain P1 from the Milos enrichment culture and "*Acidithiomicrobium*" strain P2 from the second culture (Davis-Belmar and Norris 2009). "*Acidithiomicrobium*" strain P2 was referred to as "*Acidimicrobium*" sp. 2 (Cleaver et al. 2007) before its isolation into pure culture.

Cultures (500 ml) of *Acidithiobacillus caldus* (DSM 8584) and "*Acidithiomicrobium*" strain P2 were grown in shaken flasks with elemental sulfur as substrate (5 g l^{-1}) in a medium (Norris et al. 1996) initially at pH 3. Sulfur particles were settled out of culture samples before cells remaining in suspension were measured as total particle volume by orifice electrical flow impedance using a CellFacts Particle Size Analyser (CellFacts Instruments, Coventry, UK). Sulfate was determined by atomic absorption spectrophotometry of residual barium in solution after culture supernatants were treated with barium chloride to precipitate sulfate, with reference to calibration curves obtained from precipitation of sulfate standards.

Ore leaching columns

These were operated essentially as described previously (Davis-Belmar et al. 2007), except for the temperature which was maintained at 47–48°C. Feed solution contained $(NH_4)_2SO_4$ (20 mg l^{-1}), $MgSO_4{\cdot}7H_2O$ (40 mg l^{-1}) and K_2HPO_4 (10 mg l^{-1}) and was applied at 150 ml day^{-1} (equivalent to a surface application rate of 4 l m^{-2} h^{-1}). Ore (0.7 kg per column) from the Escondida mine in Chile was leached on three occasions: in 2003 when ore fragments in the size range 0.15–0.6 g with a mean weight of 0.3 g were used (2003a in Table 2); in 2003 when ore fragments in the size range 0.9–2.4 g with a mean weight of 1.53 g were used (2003b in Table 2); in 2006 when fragments with a mean weight of 1.46 g were used (Table 2). The ore contained 0.65% (w/v) copper, 2.82% (w/v) iron and 2.18% (w/v) sulfur. Almost half of the copper was in chalcopyrite, with the remainder mostly in chalcocite, covellite and bornite. Polymetallic ore (0.7 kg) from the Talvivaara mine in Finland was leached in 2008 (Table 2): ore fragments with a mean weight of 1.01 g contained 0.31% (w/v) nickel and 0.58% (w/v) zinc, found mainly in pentlandite and sphalerite, respectively. All ore columns were inoculated with a pyrite enrichment (see above) that was established with samples from several geothermal sources (Cleaver et al. 2007).

DNA analyses

DNA was extracted from three sources: a sample of the geothermal soil sample which was used to establish the Milos pyrite enrichment culture (see above); from this 58°C Milos pyrite enrichment culture; and from ore fragments (30 g samples) taken from ore leaching columns. The extraction procedure (lysozyme, freeze–thaw cycles, phenol, and phenol–chloroform treatments) and construction of 16S rRNA gene clone banks were described previously (Burton and Norris 2000). RFLP patterns of cloned sequences were obtained with *Rsa*I and *Sau*IIIA or *Hae*II digests and representatives of different RFLP groups were sequenced.

The *cbbL* gene sequences of "*Acidithiomicrobium*" strain P2 were obtained from its draft genome (unpublished data).

Phylogenetic analyses used PHYLIP programmes Dnadist and Protdist with F84 and JTT substitution models, respectively, and the Fitch distance matrix programme (Felsenstein 2009).

mol% G+C contents of DNA from sulfur-grown cells were estimated from melting temperature analyses as described previously (Norris et al. 1996).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences determined in this study and included in phylogenetic trees have been submitted to the GenBank database under accession numbers GQ225720 ("*Acidithiomicrobium*" strain P1), GQ225721 ("*Acidithiomicrobium*" strain P2), HM057165 (clone CD34) and HM057166 (clone CD42). The *cbbL* gene sequences of "*Acidithiomicrobium*" strain P2 have been submitted with accession numbers GQ225725 (*cbbL-1*) and GU166292 (*CbbL-2*).

Results and discussion

Source, isolation and phylogeny of novel strains

DNA was extracted from the surface (0–2 cm depth) of a sulfur-rich, acidic, geothermal soil of the island of Milos. The soil sample was at pH 2 and between 55 and 60°C. 16S rRNA gene sequences were cloned from the DNA (Table 1). Three sequences which represented putative actinobacteria were compared with related sequences, many of which have been attributed to uncultured *Acidimicrobidae* (Fig. 1). Two of the three sequences (clones CD34 and CD42) were relatively distantly related to that of *Acidimicrobium ferrooxidans* and the other species of the *Acidimicrobiaceae* that oxidize ferrous iron (Fig. 1). The relative positions of clones CD34 and CD42 varied with different analysis methods, each time with very low bootstrap values, but they were always placed among sequences which included examples from bacteria of soil and an

Table 1 16S rRNA gene clone banks derived from DNA extracted from an acidic geothermal soil sample from Milos and from a 58°C pyrite enrichment culture established with the soil sample

Clone	% of clones[a]	Top BLAST search results	GenBank no.	% identity	Proposed species or taxonomic group
Bacterial sequences: geothermal soil sample					
CD29	39	*Acidithiobacillus caldus*	DQ470072	99.9	*Acidithiobacillus caldus*
CD74	28	Uncultured bacterium clone	EU419131	99.4	*Acidimicrobiaceae*
		"*Acidithiomicrobium*" strain P1	GQ225720	100	"*Acidithiomicrobium*" strain P1
CD66	15	Uncultured "*Acidithiobacillus*" strain V1	AF339743	95.9	*Acidithiobacillus* sp.
CD42	8	*Acidimicrobiaceae* bacterium IC-180	AB517669	99.9	*Acidimicrobiaceae*
CD34	7	Uncultured bacterium clone	EU419189	99.4	*Acidimicrobiales*
CD33	2	Uncultured bacterium clone	EU419182	97.0	*Verrucomicrobia* ("*Methylacidiphilales*")
CD49	1	Uncultured bacterium clone	EU419148	99.4	*Sulfobacillus* sp.
Archaeal sequences: geothermal soil sample					
CD32	61	Clone ant h4	DQ303256	96.8	*Acidiplasma* or *Ferroplasma* sp.
CD11	32	*Acidianus brierleyi*	X90477	99.5	*Acidianus brierleyi* or
		Acidianus tengchongensis	AF226987	99.5	*Acidianus tengchongensis*
CD24	3	Uncultured thermal soil archaeon	AF391990	99.6	*Crenarchaeota*
CD48	3	*Sulfolobus metallicus*	X90479	99.6	*Sulfolobus metallicus*
CD27	1	Uncultured archaeon	AF523936	97.9	*Thermoplasmatales*
Bacterial sequences: pyrite enrichment					
≡CD74	90	Uncultured bacterium clone	EU419133	99.4	*Acidimicrobiaceae*
		"*Acidithiomicrobium*" strain P1	GQ225720	100	"*Acidithiomicrobium*" strain P1
CD61	8	*Sulfobacillus acidophilus*	AY007665	91.4	*Sulfobacillus* sp.
≡CD42	2	*Acidimicrobiaceae* bacterium IC-180	AB517669	99.9	*Acidimicrobiaceae*
Archaeal sequences: pyrite enrichment					
≡CD11	100	*Acidianus brierleyi*	X90477	99.5	*Acidianus brierleyi*
		Acidianus tengchongensis	AF226987	99.5	*Acidianus tengchongensis*

"*Acidithiomicrobium*" strain P1 was isolated from the source sample of clone CD74

[a] Bacterial clone banks comprised 99 soil sample clones or 58 enrichment culture clones. Archaeal clone banks comprised 78 soil sample clones or 50 enrichment culture clones

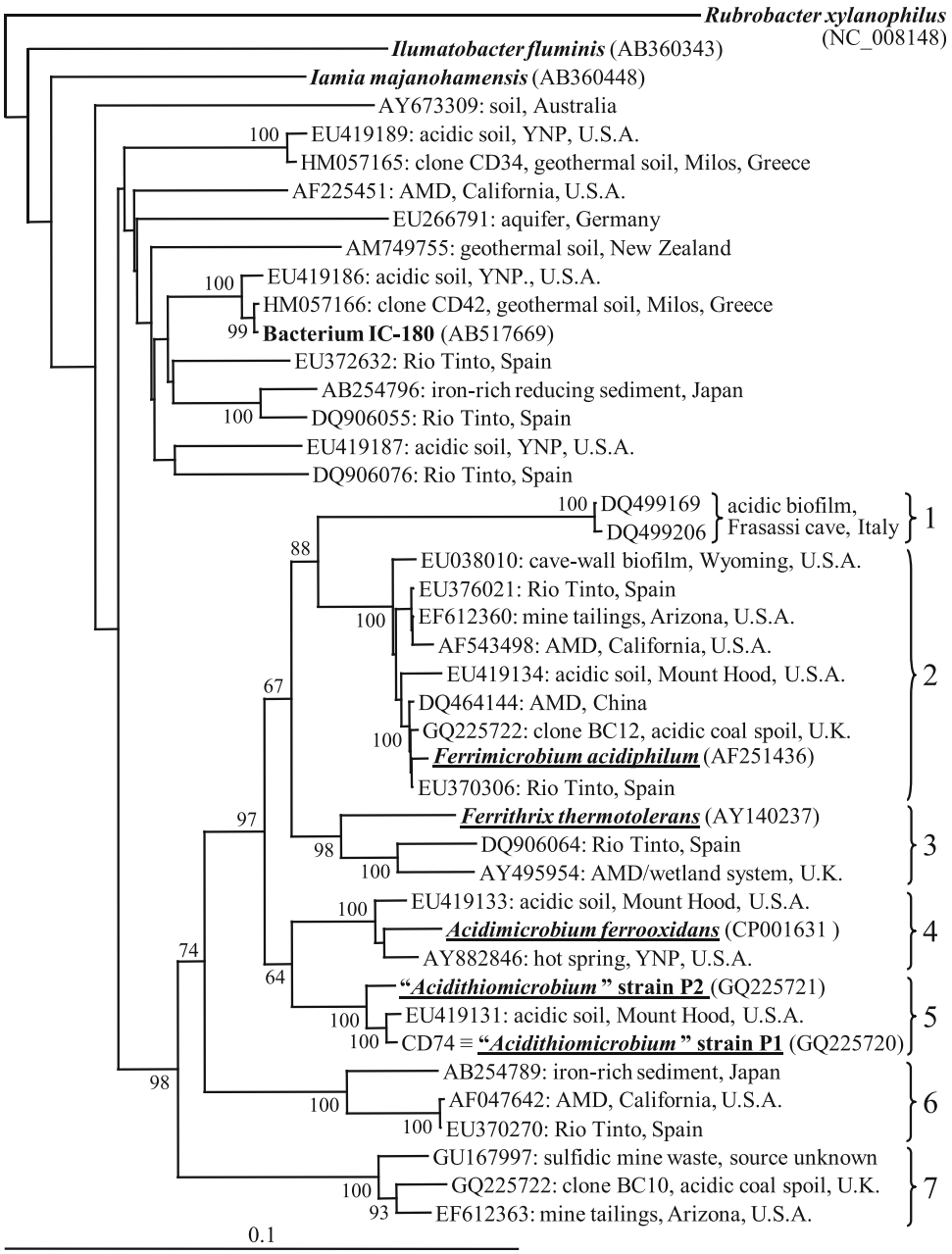

Fig. 1 Evolutionary distance tree of 16S rRNA gene sequences (1,284 aligned nucleotides) from *Rubrobacter xylanophilus* (the out-group), selected actinobacteria (*bold type*) and related sequences from uncultured "*Acidimicrobidae*". Ferrous iron-oxidizing species are *underlined*. The *scale bar* indicates 0.1 substitutions per site. Bootstrap values of 60 or greater from 100 replicates are shown (*YNP* Yellowstone National Park, *AMD* acid mine drainage)

aquifer which were much less acidic than the environments associated with the proven ferrous iron-oxidizing actinobacteria. Similarly, the *Iamia* and *Ilumatobacter* species which are represented in this region of the tree are from marine environments and are not acidophiles (Kurahashi et al. 2009; Matsumoto et al. 2009). However, an organism with a 16S rRNA gene sequence corresponding to that of clone CD42 was isolated from the same soil that provided this cloned sequence. This bacterium was acidophilic with optimum growth at about pH 2.5 and 45–50°C with various organic substrates, including glucose and xylose (Norris, unpublished data). It also appeared to have some limited capacity for oxidation of elemental sulfur during heterotrophic growth. An almost identical 16S rRNA gene sequence to that of CD42 (Table 1; Fig. 1) belongs to a novel, iron-reducing, thermoacidophilic actinobacterium from a solfatara field in Japan (Itoh et al. unpublished: GenBank AB517669).

16 rRNA gene sequences that are related more closely than CD34 and CD42 to that of *Acidimicrobium ferrooxidans* are found in several sub-groups or clusters (Fig. 1). Seven of these clusters (arbitrarily assigned numbers 1–7, Fig. 1) were similarly grouped with DNA distance, parsimony and maximum likelihood phylogenetic analyses (data not shown). The four, proven, ferrous iron-oxidizing genera are represented in four of these seven clusters

which contain sequences from geographically widespread acidic environments, with the exception of cluster one with sequences from an Italian cave (Macalady et al. 2007). The prominence of a few locations among the known sources of these sequences reflects to some extent the intensity of work at sites such as Yellowstone National Park, Iron Mountain in the USA (Druschel et al. 2004) and the Rio Tinto in Spain, where water, sediment and rhizospheres adjacent to the river have been sampled (Garrido et al. 2008; Mirete et al. 2007). Sequence CD74 which was found in the Milos geothermal soil sample was also the prevalent bacterial sequence amplified from the 58°C pyrite enrichment culture which was established with the soil sample (Table 1). An organism with this rRNA gene sequence was subsequently isolated from the enrichment culture. It was shown to grow on sulfur (see later) and is referred to as "*Acidithiomicrobium*" strain P1 (cluster 5, Fig. 1). "*Acidithiomicrobium*" strain P2 is another bacterium with a 16S rRNA gene sequence related to that of *Acidimicrobium ferrooxidans* and particularly to that of "*Acidithiomicrobium*" strain P1 (Fig. 1). This strain P2 was isolated from a pyrite enrichment culture which was established with samples from several sites, which included natural geothermal environments and mineral sulfide and coal mines (Cleaver et al. 2007). An rRNA gene sequence closely related to those of the "*Acidithiomicrobium*" strains was recovered from volcanic soil in the USA (GenBank EU419131: cluster 5, Fig. 1) and samples from geothermal sites in Iceland, Montserrat and New Zealand harboured bacteria resembling the "*Acidithiomicrobium*" strains (A. Cleaver and P. Norris, unpublished data).

The optimum growth temperatures of the "*Acidithiomicrobium*" strains are about 50°C. The mol% G+C contents of their DNA are 55% (strain P1) and 51% (strain P2), in the same range as those of *Ferrimicrobium acidiphilum* (cluster 2, Fig. 1; 55 mol% GC) and *Ferrithrix thermotolerans* (cluster 3, Fig. 1; 50 mol% GC), respectively. The 68 mol% G+C content of *Am. ferrooxidans*, therefore, appears exceptional among these acidophiles and is 17% higher than that of "*Acidithiomicrobium*" strain P2, one of its closest relatives with which it shares 96% 16S rRNA gene sequence identity.

Other cloned bacterial rRNA gene sequences from the Milos geothermal site (Table 1) were CD33, which was related to the acidophilic, thermotolerant, methane-oxidizing bacteria (Dunfield et al. 2007; Islam et al. 2008); CD29 from the thermotolerant, sulfur-oxidizing proteobacterium *Acidithiobacillus caldus*; CD66 from an uncultured *Acidithiobacillus* species; and CD49 from an uncultured species related to those of the *Sulfobacillus* genus (ferrous iron- and sulfur-oxidizing, acidophilic Firmicutes).

The Milos geothermal soil was also the source of several cloned rRNA sequences representing thermophilic acidophiles of the Euryarchaeota and Crenarchaeota (Table 1). One of these organisms, an *Acidianus* species, was represented in the pyrite enrichment culture from which "*Acidithiomicrobium*" strain P1 was isolated (Table 1). Microscopy indicated similar numbers of *Acidianus*-like and "*Acidithiomicrobium*"-like organisms in the enrichment culture at 58°C.

Autotrophy

"*Acidithiomicrobium*" strains P1 and P2 were observed to grow in pure culture in the absence of any organic supplements. *Acidimicrobium ferrooxidans* has previously been observed to grow autotrophically with ferrous iron as substrate but this growth was limited (Clark and Norris 1996) and its growth autotrophically with pyrite as substrate was extremely poor (Davis-Belmar and Norris 2009). In contrast, strong autotrophic growth of the "*Acidithiomicrobium*" strains occurred with ferrous iron or pyrite as substrate (Davis-Belmar and Norris 2009).

The similarity of the amino acid sequence of the RuBisCO large subunit of *Acidimicrobium ferrooxidans* to that of the proteobacterium *Acidithiobacillus ferrooxidans* was noted when RuBisCO-dependent CO_2 fixation by the former was observed (Clark 1995). Form I RuBisCOs with eight large (CbbL) and eight small (CbbS) subunits have been labelled green or red types with reference to their presence in chlorophytes or red algae (Delwiche and Palmer 1996). Both types, and two major sub-groups of each (groups A and B and groups C and D), were noted in prokaryotes (Watson and Tabita 1997). A fifth group has been proposed (Park et al. 2009) to include CbbLs of the phototroph *Oscillochloris trichoides* (Tourova et al. 2006), the acidophilic Firmicute *Sulfobacillus acidophilus* (Caldwell et al. 2007) and a *Mycobacterium* species (Park et al. 2009). Two different form IA enzymes in many autotrophic proteobacteria, including *Acidithiobacillus ferrooxidans*, have been referred to as IAc and IAq types (Badger and Bek 2008), depending on whether carboxysome genes or *cbbQ* genes are immediately downstream of *cbbL* and *cbbS*. The *cbbQ* gene product is thought to be involved in post-translational regulation of RuBisCO. Carboxysomes compartmentalize RuBisCO and have carbonic anhydrase activity to promote efficient CO_2 fixation (Cannon et al. 2002). The RuBisCO genes in *Am. ferrooxidans* are adjacent to carboxysome-encoding genes (GenBank accession CP001631). Immediately downstream of these are several ORFs encoding proteins that are most closely related to those of *At. ferrooxidans*. This region of 16–17 kbp DNA could have been acquired from *At. ferrooxidans* or its ancestor. CbbL proteins with highest

identities to the proteins of *Am. ferrooxidans* and *At. ferrooxidans* are also found in "*Acidithiomicrobium*" strains P2 (Fig. 2) and P1 (not shown). It is so far unknown why the autotrophic capacity of *Am. ferrooxidans* appears much less than that of the other two species. In contrast to *At. ferrooxidans* and *Am. ferrooxidans*, "*Acidithiomicrobium*" strain P2 has also *cbbL*/*cbbS* genes which could encode a form ID red-type enzyme (Fig. 2).

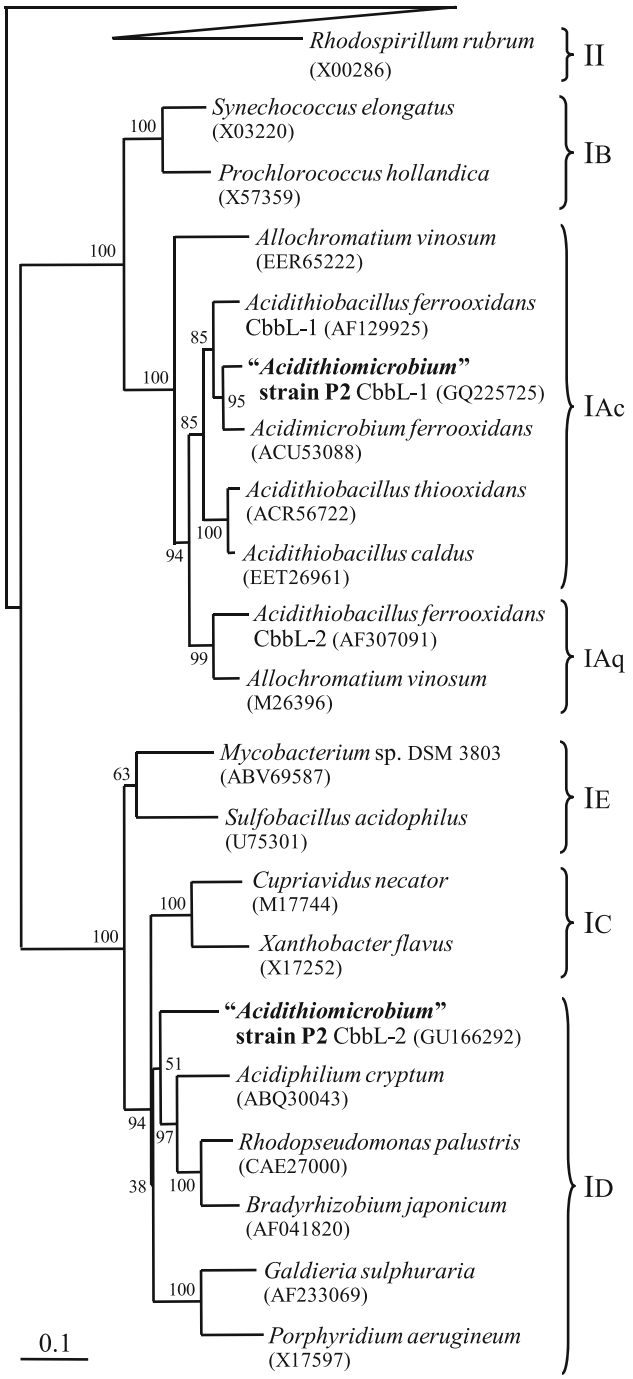

Fig. 2 Distance tree of RuBisCO form I large subunit partial sequences (459 aligned amino acids) and a form II RuBisCO large subunit (the out-group). The *scale bar* indicates 0.1 substitutions per site. Bootstrap values from 100 replicates are shown

Growth on sulfur

The proteobacterium *Acidithiobacillus caldus* (Norris et al. 1986; Hallberg and Lindström 1994) is generally considered to be the most active acidophilic, thermotolerant, sulfur-oxidizing bacterium in mineral leaching environments. The extent of sulfur oxidation by "*Acidithiomicrobium*" strain P2 was similar to that by *At. caldus* (Fig. 3), with acidity increased to pH 1.4 at the end of exponential phases of growth in batch cultures. Biomass which might be attached to sulfur was not measured, but growth of strain P2 cells in terms of planktonic cells appeared at least as extensive as that of *At. caldus*. Growth of "*Acidithiomicrobium*" strain P1 on sulfur was highly similar to that of strain P2 at both 47 and 52°C (data not shown). This capacity for sulfur oxidation was unknown when strain P2 (then referred to as "*Acidimicrobium* sp. 2") was not available in pure culture and was recognized only by fluorescent in situ hybridization with a specific rRNA gene probe (Cleaver et al. 2007). Sulfur oxidation could have contributed to its competitive advantage over the mineral

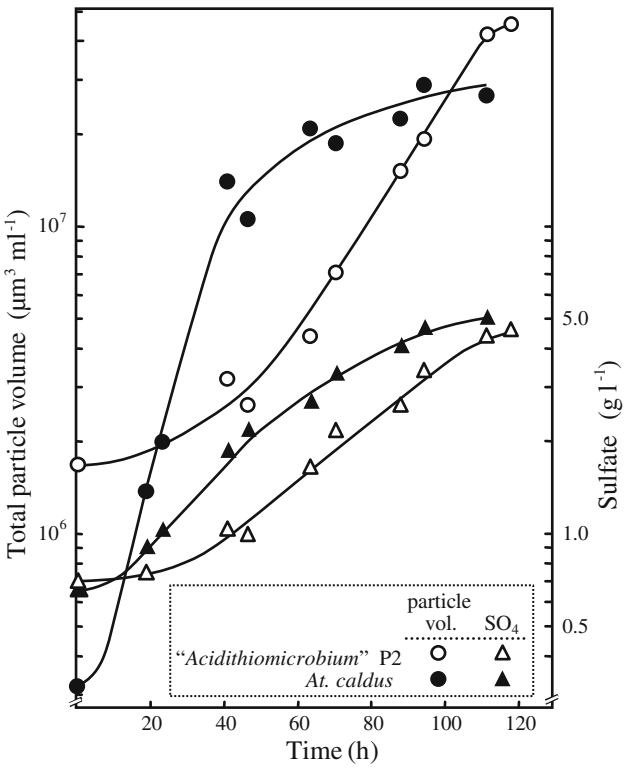

Fig. 3 Autotrophic growth of "*Acidithiomicrobium*" strain P2 (at 52°C) and *Acidithiobacillus caldus* (at 47°C) on elemental sulfur. Total particle volume corresponds to planktonic biomass (cells unattached to sulfur particles)

sulfide-oxidizing *Am. ferrooxidans* and *Sulfobacillus thermosulfidooxidans* in a mixed culture that extracted nickel from a mineral sulfide at 47°C in bioreactors (Cleaver et al. 2007). Strong autotrophic growth of *S. thermosulfidooxidans* or *Am. ferrooxidans* on sulfur has not been observed and *Am. ferrooxidans* causes only slight acidification of the medium when grown heterotrophically on yeast extract in the presence of sulfur (Clark and Norris 1996).

"*Acidithiomicrobium*" in mineral sulfide ore leaching

A potential role for "*Acidithiomicrobium*"-like bacteria in ore leaching was assessed with laboratory columns containing ores from the Talvivaara mine in Finland and the Escondida mine in Chile. Leaching of a constructed ore heap (0.8 km × 2.4 km, 8 m height) began at Talvivaara in 2008. There are ore reserves for 40 years and planned annual production of over 100,000 tonnes of a mixture of nickel, zinc, copper and cobalt (M. Riekkola-Vanhanen, personal communication). Leaching of low grade sulfide ore reserves of about 1,500 million tonnes began at Escondida in 2006 with current annual production of about 200,000 tonnes of copper from ore heaps with a surface area of about 5 km^2. Ore leaching columns were inoculated at 47°C with the mixed culture that was the source of "*Acidithiomicrobium*" strain P2 (Cleaver et al. 2007). DNA was extracted from a Talvivaara ore column after 130 days, when 17% of the nickel and 14% of the zinc had been leached. DNA was extracted from three Escondida ore columns after 70 days operation when 56% of the copper had been leached (2003a, Table 2), after 90 days when 36% of the copper had been leached (2003b, Table 2) and after 74 days when 62% of the copper had been leached (2006, Table 2). This discussion focuses on the microorganisms present in the ore columns with the influence of the ore fragment size and other operational parameters on the metal extraction to be described elsewhere.

"*Acidithiomicrobium*" strain P2 sequences dominated small clone banks of 16S rRNA genes derived from DNA extracted from all four columns (Table 2). A 16S rRNA gene sequence identical to that of "*Acidithiomicrobium*" strain P2 was recovered from a mineral sulfide-processing mixed culture that shared source material with the enrichment culture used in this work (see Cleaver et al. 2007) and was previously deposited in GenBank (uncultured bacterium strain JTC04, accession no. AY805539; Table 2). These small clone banks were intended to indicate the major organisms in the ore columns, rather than reflect the full diversity of the microbial populations. *Acidithiobacillus caldus* was prominent in the enrichment culture inocula for the ore columns and its 16S rRNA gene sequence was amplified by PCR from the DNA extracted from Escondida ore columns with *At. caldus*-specific primers, although it was not recorded in these column clone banks. Dot blots of RNA extracted directly from the ore samples confirmed its presence at a relatively very low cell number compared to the actinobacteria (data not shown). These dot blots carried out with RNA from the

Table 2 16S rRNA gene clone banks derived from DNA extracted from ore columns (47°C) inoculated with a moderately thermophilic pyrite enrichment culture

Clone[a]	Escondida ore			Talvivaara ore	Top BLAST search results	GenBank no.	% identity
	2003a	2003b	2006	2008			
Bacterial sequences[b]							
LCB1	85	98	100	50	Uncultured bacterium clone JTC04	AY805539	100
					"*Acidithiomicrobium*" strain P2	GQ225721	100
LCB37	13	2	0	31	*Acidimicrobium ferrooxidans*	CP001631	100
CFB46	0	0	0	18	*Acidithiobacillus caldus*	NR_026517	99.9
LCB4	2	0	0	1	*Sulfobacillus thermosulfidooxidans*	GU180244	99.7
Archaeal sequences[c]							
CD1	–[d]	–	88	50	Uncultured archaeon clone ant g4	DQ303254	98.2
					Uncultured archaeon clone ASL1	AF544224	98.1
CD16	–	–	11	50	*Ferroplasma cupricumulans*	AY907888	99.9
CD2	–	–	1	0	Uncultured archaeon clone ASL1	AF544224	96.2

Numbers are % of total clones in each clone bank. "*Acidithiomicrobium*" strain P2 was isolated from the enrichment culture that was the source of the ore column inocula

[a] The code for the first clone identified is shown where clones with identical sequences were found in different ore columns

[b] Bacterial clone banks comprised 48 (2003a), 48 (2003b), 62 (2006) and 90 (2008) clones

[c] Archaeal clone banks comprised 63 (2006) and 34 (2008) clones

[d] No attempt was made to clone archaeal sequences

Escondida ore columns operated in 2003 also indicated approximately equal amounts of "*Acidithiomicrobium*" strain P2 and *Acidimicrobium ferrooxidans* in the leached ore samples, although the clone bank analysis suggested dominance of strain P2.

Two archaeal 16S rRNA gene sequences were prevalent in the clone banks derived from DNA extracted from the ore columns (Table 2). One of these (CD1) represented an uncultured strain predicted by phylogenetic analysis to be related to ferrous iron-oxidizing, acidophilic euryarchaea (data not shown). The closest related sequences were previously cloned from samples of acid mine drainage in the USA (clone ASL1: Baker and Banfield 2003) and Spain (clone ant g4: García-Moyano et al. 2007). The second sequence (CD16) was highly similar to that of moderately thermophilic strains of ferrous iron-oxidizing euryarchaea. This sequence was also recovered from a commercial bioreactor processing a gold-bearing mineral sulfide from the Fairview mine in South Africa (noted in Burton and Norris 2000). It was also attributed to *Ferroplasma cupricumulans* (now *Acidiplasma cupricumulans*), which was abundant in an ore biomining operation in Myanmar (Hawkes et al. 2006), and to *Acidiplasma aeolicum*, which was isolated from the island of Vulcano, Italy (Golyshina et al. 2009). It seems likely that some release of organic carbon following CO_2 fixation by "*Acidithiomicrobium*" strain P2 in the ore columns could have supported the growth of these lithotrophic euryarchaea, in which autotrophy has not been proven (Dopson et al. 2004).

Successful application of these novel actinobacteria remains to be proven in commercial ore heap leaching. The benefits of thermophile activity in ore heaps would be the more efficient extraction of metals that can occur at elevated temperatures (Petersen and Dixon 2002) and continued bioleaching in heap zones where the exothermic oxidation of minerals inevitably increases the temperature, as in the Talvivaara ore heap (M. Riekkola-Vanhanen, personal communication). The mechanisms by which the mineral sulfide-oxidizing actinobacteria oxidize ferrous iron and sulfur and expression of the different forms of RuBisCO in "*Acidithiomicrobium*" under different conditions remain to be established.

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